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Enzymes of *Trichomonas foetus*

THE ACTION OF CELL-FREE EXTRACTS ON BLOOD-GROUP SUBSTANCES AND LOW-MOLECULAR-WEIGHT GLYCOSIDES

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Enzymes which destroy the serological activity of the water-soluble blood-group substances have been isolated from many sources (see Kabat, 1956). In those instances where chemical studies have been made the loss of serological specificity was accompanied by the liberation of reducing sugars, indicating that the enzymes are breaking down the carbohydrate portion of the mucopolysaccharide molecules. Thus enzyme preparations from snail (Busycon)-liver extract liberate methylpentose and *N*-acetylhexosamine from hog A-substance (Howe & Kabat, 1953) and preparations from *Clostridium welchii* set free fucose, *N*-acetylhexosamine and galactose from hog and human H-substances (Crumpton & Morgan, 1953; Buchanan, Crumpton & Morgan, 1957). György, Rose & Springer (1954) similarly showed that an enzyme preparation from *Lactobacillus bifidus* var. *Penn.* liberates fucose, *N*-acetylhexosamine and galactose from blood-group substances prepared from ovarian cysts and meconium of all A, B, O blood groups. An interpretation of the changes in the structure of the blood-group mucopolysaccharides brought about by these enzymes is rendered difficult by the extent of disruption of the molecules and the similarity of the enzymic hydrolysis products irrespective of the blood group of the individual from whom the starting material was derived. It would appear that the enzyme preparations contain a mixture of enzymes: hexosaminidases, fucosidases, galactosidases and possibly peptidases, which bring about further breakdown of the molecule after the initial change, which leads to loss of specificity, has occurred. The presence of *N*-acetyl-α- and -β-glucosaminidases in the snail-liver preparations used by Howe & Kabat (1953) has been demonstrated and the preparations from *L. bifidus* var. *Penn.* were known to contain an *N*-acetyl-β-glucosaminidase and a β-galactosidase (György *et al.* 1954), but in no instance was the specificity of the

enzymes which destroy the serological activity of the blood-group substances directly determined.

In an attempt to find a different source of enzyme which would decompose the specific water-soluble blood-group materials, saline extracts of living cultures of certain protozoa were examined (Watkins, 1953) and it was found that extracts of the flagellate *Trichomonas foetus* rapidly destroyed the serological activity of the human A-, B-, H-, Le^a- and Le^b-substances. The results of enzyme-inhibition tests with simple sugars, taken in conjunction with the results of agglutination-inhibition experiments, led to the suggestion (Watkins & Morgan, 1955*a*) that the enzymes in *T. foetus* extracts responsible for the loss of A, B and H serological activity are respectively an *N*-acetylgalactosaminidase, a galactosidase and a fucosidase. Direct confirmation of these specificities must wait until enzyme preparations have been obtained of such a degree of purity that the only chemical changes induced by them in the blood-group molecules are those which result from the loss of serological specificity. Purification procedures on the *T. foetus* enzymes are now being investigated with this aim in view. The results described in the present paper on the occurrence and properties, in cell-free extracts from *T. foetus*, of the enzymes which act on the blood-group substances and of enzymes which hydrolyse low-molecular-weight substrates known to contain galactosyl, *N*-acetylhexosaminoyl and fucosyl structures are intended as a basis for these attempts to separate and purify the enzymes.

MATERIALS

All melting points recorded are uncorrected.

Organism culture. Cultures of *T. foetus* var. Belfast were kindly supplied by Dr Muriel Robertson, F.R.S. The organisms were grown in pure culture for 48 hr. at 37° in 1 l. quantities in a glucose–serum broth medium (Kerr & Robertson, 1947). The organisms reached a density of about 5×10^6 cells/ml.

Specific blood-group substances. A-, B-, H-, and Le^a-substances isolated from ovarian-cyst fluids by methods described previously (Aminoff, Morgan & Watkins, 1950; Annison & Morgan, 1952*a*, *b*; Gibbons & Morgan, 1954; Gibbons, Morgan & Gibbons, 1955) were kindly supplied by Professor W. T. J. Morgan, F.R.S.

o-*Nitrophenyl* α- *and* β-D-*galactosides.* The α-compound, prepared by Dr W. R. C. Crimmin according to the method described by Porter, Holmes & Crocker (1953), had m.p. 145° (literature, m.p. 145–147°). The β-compound was a commercial preparation obtained from L. Light and Co. Ltd.

p-*Nitrophenyl* N-*acetyl*-β-D-*glucosaminide.* This was prepared by Dr W. R. C. Crimmin according to the method of Westphal & Schmidt (1952). It had m.p. 214° (lit., m.p. 214°).

p-*Nitrophenyl* N-*acetyl*-β-D-*galactosaminide.* This was supplied by Dr H. Feier and had m.p. 220–222°, $[\alpha]_D$ −35·4° in pyridine. The compound had been prepared by the method described for the β-glucosaminide (Westphal & Schmidt, 1952).

p-*Nitrophenyl* α-L-*rhamnoside and* p-*nitrophenyl* α-D-*mannoside.* These were kindly supplied by Dr Feier and had been prepared by the methods described by Westphal & Feier (1956).

Methyl N-*acetyl*-α- *and* -β-D-*glucosaminides.* These were prepared by Dr W. R. C. Crimmin as by Zilliken, Rose, Braun & György (1955*b*). The α-compound had m.p. 188–190°, $[\alpha]_D$ +127° in water (lit., m.p. 188°, $[\alpha]_D$ +131·5°) and the β-compound had m.p. 191–193°, $[\alpha_D]$ −41° in water (lit., m.p. 204°, $[\alpha]_D$ −44·3°).

Methyl N-*acetyl*-α-*galactosaminide.* This was prepared by Dr W. R. C. Crimmin as by Zilliken *et al.* (1955*b*) for the preparation of methyl *N*-acetyl-α-glucosaminide. It had m.p. 200°, $[\alpha]_D$ +160° (lit., m.p. 217°, $[\alpha]_D$ +170°).

O-α-D-*Galactopyranosyl*-(1→6)-D-*galactose and* O-β-D-*galactopyranosyl*-(1→6)-D-*galactose.* These were prepared and kindly supplied by Drs Whelan and Feier. The α-compound had $[\alpha]_D$ +135° in water (lit., $[\alpha]_D$ +149°) and the β-compound $[\alpha]_D$ +32·9° in water (lit., $[\alpha]_D$ +34·1°).

O-α-D-*Galactopyranosyl*-(1→6)-N-*acetyl*-D-*glucosamine and* O-α-D-*galactopyranosyl*-(1→6)-N-*acetyl*-D-*galactosamine.* These were prepared by enzymic synthesis as described previously (Watkins, 1958).

O-β-D-*Galactopyranosyl*-(1→3)-N-*acetyl*-D-*glucosamine and* O-β-D-*galactopyranosyl*-(1→6)-N-*acetyl*-D-*glucosamine.* These were obtained through the kindness of Professor R. Kuhn.

O-β-D-*Galactopyranosyl*-(1→4)-N-*acetyl*-D-*glucosamine.* This was kindly supplied by Professor György.

Disaccharides from fucoidin. Three disaccharides which had been isolated from fucoidin (R. H. Côté, unpublished results) were kindly supplied by Dr Côté; *O*-α-L-fucopyranosyl-(1→2)-L-fucopyranose had $[\alpha]_D$ −169° in water; *O*-α-fucopyranosyl-(1→3)-L-fucopyranose had $[\alpha]_D$ −191° in water and *O*-α-L-fucopyranosyl-(1→4)-L-fucopyranose had $[\alpha]_D$ −170° in water.

Oligosaccharides from milk. *O*-α-L-Fucopyranosyl-(1→2)-D-galactopyranose (Kuhn & Gauhe, 1958*a*), *O*-α-L-fucopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranose (fucosidolactose; Kuhn, Baer & Gauhe, 1955), *O*-α-L-fucopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-(1→3)-*N*-acetyl-*O*-β-D-glucosaminopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranose (lacto-*N*-fucopentaose I; Kuhn, Baer & Gauhe, 1956), *O*-β-D-galactopyranosyl-(1→3)-*O*-[α-L-fucopyranosyl-(1→4)-]-*N*-acetyl-*O*-β-D-glucosaminopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranose (lacto-*N*-fucopentaose II; Kuhn, Baer & Gauhe, 1958), *O*-α-L-fucopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-(1→4)-*O*-[α-L-fucopyranosyl-(1→3)]-D-glucopyranose (lactodifucotetraose, Kuhn & Gauhe, 1958*b*) were made available through the kindness of Professor R. Kuhn.

N-*Acetyl*-O-β-D-*glucosaminopyranosyl*-(1→4)-N-*acetyl*-D-*glucosaminopyranose* (*NN'*-diacetylchitobiose). This was prepared by Dr W. R. C. Crimmin by the method described by Zilliken, Braun, Rose & György (1955*a*). It had m.p. 250°; $[\alpha]_D$ +18° in water (lit., m.p. 245–247°; $[\alpha]_D$ +18·5°).

Buffers. In the pH range 3·0–5·4 mixtures of 0·2M-Na_2HPO_4 and 0·1M-citric acid (McIlvaine, 1921) were used, in the pH range 5·6–8·0 mixtures of 0·066M-Na_2HPO_4 and 0·066M-KH_2PO_4 (Sørensen, 1909), and in the pH range 8·5–11 mixtures of 0·1M-glycine, 0·1M-NaCl and 0·1M-NaOH (Walbum, 1920).

METHODS

Preparation of cell-free extracts of T. foetus. The washed living organisms from 1 l. of culture fluid, suspended in 10 ml. of physiological saline (0·15 M-NaCl), were lysed by freezing three times at −80°. The cellular debris was removed by centrifuging at 4000 *g* on a high-speed Servall centrifuge for 1 hr. at 4°. The supernatant liquid was filtered through a bacteriological filter candle and the clear filtrate was either tested directly for enzyme activity or dialysed for 48 hr. against frequent changes of distilled water and dried from the frozen state.

Serological activity. Isoagglutination-inhibition and haemolysis-inhibition tests for A-activity were carried out as described by Morgan & King (1943). The B-substance was tested for inhibition with natural human isoagglutinins and immune rabbit anti-B agglutinins as described by Gibbons & Morgan (1954). A technique similar to that described by Grubb & Morgan (1949) for Le^a-inhibition tests was used for the determination of H and Le^a serological activity. A human anti-Le^a serum was used for the Le^a tests and an immune rabbit anti-H serum (Morgan & Waddell, 1945) was used for the majority of the H-inhibition tests. Precipitin tests were carried out as described previously (Morgan & Watkins, 1951) with immune rabbit precipitating sera. The precipitating antibodies were induced by artificial antigens made by combining A-, B-, H- or Le^a-substances with the conjugated protein of the somatic O antigen of *Shigella dysenteriae* by the method described by Morgan (1943).

Liberation of o- *and* p-*nitrophenol.* Hydrolysis of the nitrophenyl glycosides was followed by a method essentially similar to that described by Cohn & Monod (1951). The change in optical density at 400 mμ was measured in a Unicam SP. 600 spectrophotometer. Readings were taken at intervals of 1 min. for a total of 5 min. The reactions were carried out at room temperature in 66 mM-phosphate buffer (pH 7·0) unless otherwise stated. Substrate concentrations of 10 mM were employed unless the solubility of the nitrophenyl glycoside was lower than this concentration, when saturated solutions were used.

Chromatography. Chromatograms were run on Whatman no. 4 paper in butanol–pyridine–water (6:4:3, by vol.) solvent mixture by the use of the ascending technique. Benzidine–trichloroacetic acid spray reagent (Bacon & Edelman, 1951) was used for detecting reducing sugars and the method of Partridge (1948) for detecting acetylhexosamines.

Reducing sugars. These were determined by the colorimetric technique of Nelson (1944). Reducing values were expressed in terms of a glucose standard.

N-*Acetylhexosamine.* This was assayed by the method of Aminoff, Morgan & Watkins (1952).

RESULTS

Examination of Trichomonas foetus *extracts for enzyme activity: enzymes which act on blood-group substances*

The cell-free *T. foetus* filtrates were tested for their capacity to destroy the serological activity of the human blood-group A-, B-, H- and Le^a-substances. Solutions (0·1 %) of the blood-group substances were mixed with (*a*) an equal volume, and (*b*) with one-tenth volume, of the *T. foetus* extract and the mixtures were incubated overnight at 37° after the addition of toluene. An equal volume of the extract which had been heated for 10 min. at 100° was added to the controls. After incubation all the solutions were heated for 10 min. in a boiling-water bath to stop the enzyme action and the serological activity of the preparations was determined by means of agglutination-inhibition tests. The minimum amounts of A-, B-, H- and Le^a-substances required to give complete inhibition in the homologous agglutination system before and after treatment with extracts of *T. foetus* are given in Table 1.

Action on A-substance. The degree of inactivation of A-substance recorded in Table 1 was measured by using group-A_1 cells in the isoagglutination system. The use of red cells of the subgroup A_2 in place of A_1 cells gave similar results. The activity of the A-substance as measured by the haemolysis-inhibition test was also destroyed by treatment with the *T. foetus* preparation. The haemolysis-inhibition test is generally believed to measure the 'Forssman' or heterophile component of the A agglutinogen and is accepted as measuring a different, although closely related, serological property of the A-substance from that measured by the isoagglutination-inhibition technique. The degree of precipitation given by the A-substance with a homologous precipitating immune rabbit anti-A serum was considerably reduced after the action of the *T. foetus* enzyme preparation.

Action on B-substance. Gibbons & Morgan (1952) showed that the purified human B-substance obtained from ovarian-cyst fluids has two partial specificities. One is measured by the naturally occurring human anti-B agglutinins and is labile to treatment with mild alkali. The other, measured by immune anti-B agglutinins of human or animal origin, is stable to mild alkali treatment under carefully controlled conditions. Examination of the action of the *T. foetus* extracts on both these specificities has shown that with most enzyme preparations the activity of B-substance is reduced against both natural and immune anti-B agglutinins. The extent of inactivation is, however, usually greater when the naturally occurring anti-B agglutinins are used as test reagents. Occasionally enzyme preparations have been obtained from *T. foetus* which show considerable capacity to inactivate the property measured by the natural antibodies but which fail to produce detectable inactivation of the property measured by immune anti-B reagents. The degree of precipitation of B-substance with an immune precipitating rabbit anti-B serum after treatment with such an enzyme preparation has not been examined, but substances

Table 1. *Serological inactivation of human A-, B-, H- and Le^a-substances by cell-free extracts of* T. foetus

Blood-group substance	Test serum	Volume of *T. foetus* extract added* (ml.)	Minimum amount of blood-group substance giving complete inhibition† (μg./ml.)
A	Human anti-A	—	1
		0·1	4
		1·0	63
		1·0 (boiled)	1
B	Human anti-B	—	0·5
		0·1	16
		1·0	125
		1·0 (boiled)	0·5
H	Immune rabbit anti-H	—	2
		0·1	>500
		1·0	>500
		1·0 (boiled)	2
Le^a	Human anti-Le^a	—	1
		0·1	16
		1·0	>500
		1·0 (boiled)	1

* *T. foetus* extract added to 1 ml. of a 0·1% (w/v) solution of blood-group substance.
† Equal volume of solution containing dilution of blood-group substance added to volume of test serum containing 2–3 completely haemagglutinating doses.

treated with *T. foetus* extracts which destroy the activity with both natural and immune agglutinins also lose their ability to precipitate with the immune reagent.

Action on H-substance. Many different anti-H reagents of human, animal and plant origin have been described. These reagents all have the common property of being inhibited in their action on O cells by human H-substance, although they can be shown to differ in other respects (see Watkins & Morgan, 1955*b*). Agglutination-inhibition tests with H-substance which had been treated with *T. foetus* extract showed that the activity is destroyed with immune rabbit anti-H serum (Morgan & Waddell, 1945), natural eel anti-H serum (Sugishita 1935), natural cattle anti-H serum (Schiff, 1927), anti-H sera from chickens immunized with *Shigella dysenteriae* (Grubb, 1949) and with the human anti-H reagents 'Warboys' (Watkins, 1952) and 'Tomlinson' (Watkins & Morgan, 1954). The capacity of H-substance to precipitate with immune rabbit precipitating anti-H serum was also lost on treatment with the enzyme preparations.

Action on Le^a-substance. The destruction of the Le^a serological character could be shown both by its loss of activity in the agglutination-inhibition test (Table 1) and by its failure to precipitate with a precipitating immune rabbit anti-Le^a serum after the action of the *T. foetus* preparation.

Development of type XIV activity. Blood-group substances isolated from hog gastric mucin, human saliva and human stomachs by peptic digestion always cross-react in precipitation tests with type XIV anti-pneumococcus horse serum (Kabat, Bendich, Bezer & Knaub, 1948). It is possible, however, to select human-blood-group A-, B- and H-substances isolated from ovarian cysts by extraction with phenol (Morgan & King, 1943) which do not precipitate to any appreciable extent in this system. Treatment of these substances with the crude *T. foetus* enzyme preparations resulted in the development of considerable cross-reactivity with the type XIV antiserum.

Development of H specificity by A- and B-substances. Iseki & Masaki (1953), using an A-decomposing enzyme from *Cl. tertium*, reported that when this enzyme acts on A-substance the A specificity disappeared and O(H) specificity developed. Subsequently, Iseki & Ikeda (1956) showed that B-substance inactivated by a specific enzyme from *Bacillus cereus* also developed H-specific properties. Watkins (1956) found that when human B-substance was treated with a purified B enzyme from *T. foetus*, which was free from H enzyme, the disappearance of B activity was accompanied by a development of H specificity. The presence of an H-decomposing enzyme in the crude *T. foetus* extracts used in the present investigation means that any H activity which is formed by the action of the A- and B-decomposing enzymes on their respective substrates is immediately destroyed. However, if the inactivation is carried out in the presence of L-fucose (final concentration 2%), which inhibits the H-decomposing enzyme (Watkins & Morgan, 1955*a*), both the A- and B-substances

can be shown to develop the capacity to inhibit the agglutination of O cells by rabbit anti-H sera. The activity developed is equivalent to only 5–10 % of the activity of a purified human H-substance, but the A and B materials used were completely free from H activity before enzyme treatment. The inhibition of the Le^a-decomposing enzyme by L-fucose (Watkins & Morgan, 1957) prevented the use of this sugar to determine whether H-specific properties are developed by Le^a-substance after enzyme treatment.

Chemical changes in the blood-group substances. Incubation for 18 hr. at 37° of 1 % (w/v) solutions of A-, B-, H- and Le^a-substances with an equal volume of a 1 % (w/v) solution of a dialysed extract of *T. foetus* dissolved in phosphate buffer (pH 7) resulted in the liberation from each substance of about 20 % of reducing sugars (in terms of a glucose standard). Part of the materials became diffusible through a cellophan membrane, and chromatographic examination of the diffusible products, after removal of salts by passing the solution through a column of Bio-Deminrolit resin, showed that fucose, galactose and *N*-acetylhexosamine had been split off from each substance. No evidence was obtained for the liberation of di-, tri- or higher oligo-saccharides. In view of the large amount of protein added with the crude extract and the possibility of autolysis the diffusible products were not examined for the presence of liberated amino acids or peptides.

Action on red cells. The direct action of the crude *T. foetus* enzyme preparation on the blood-group receptors on the red-cell surface could not be determined, because exposure of red cells to the enzyme solutions results immediately in non-specific agglutination of the cell suspension and, furthermore, incubation with the *T. foetus* extract at 37° causes extensive haemolysis of the cells within 1 hr. The capacity of the extract to agglutinate cells was destroyed by heating the preparation for 10 min. at 100°, and considerably reduced, although not completely destroyed, by heating for 10 min. at 64°.

Enzyme production during the growth of Trichomonas foetus *var. Belfast.* The development of the enzymes which destroy the blood-group substances at different stages in the life cycle of *T. foetus* organisms has not been examined in detail. Comparison of extracts prepared from a 24 hr. growth and the 48 hr. growth normally used showed that the enzymes acting on A-, B-, H- and Le^a-substances were present after 24 hr. The activity on a dry-weight basis was less, however, and the total yield of dry material was only about one-fifth of the yield from a 48 hr. growth.

Activity of extracts prepared by different methods. Extracts of *T. foetus* organisms were prepared by disintegration of a saline suspension in a Waring Blendor and by freezing and thawing suspensions in (i) 50 % (v/v) glycerol, (ii) a 5 % solution of the surface-active agent Triton X 100 (Rohm and Haas Co., Philadelphia) and (iii) buffer solutions (pH 5·4, 7·0 and 9·1). The cellular debris was removed by high-speed centrifuging, and the supernatant solutions were dialysed for 48 hr. against frequent changes of distilled water, and, with the exception of the Triton X 100 extract, dried from the frozen state. The yields and enzyme activities of the preparations were compared with those of an extract prepared from approximately equal numbers of the same batch of organisms by the standard procedure of freezing and thawing a saline suspension.

The yield of dry material in the extracts obtained by disintegration of the organisms in the Blendor and by extraction with glycerol was similar to that obtained by the standard method, and the three preparations produced a similar degree of inactivation of A-, B- and H-substances. A strict comparison of the activity of the enzymes acting on the blood-group substrates in the extracts prepared with Triton X 100 could not be made because the Triton, which was not removed by dialysis, caused haemolysis of red-blood cells and thus interfered at lower dilutions in the agglutination-inhibition tests; the limited tests carried out, however, showed that A-, B- and H-decompsing enzymes were present in the preparations.

The yields of dry material extracted at pH 5·4, 7·0 and 9·1 were respectively 59, 132 and 188 % of the yield obtained by extraction with saline. The activity of the preparations against A-, B- and H-substances showed little variation, although there was some indication that the material extracted at pH 5·4 was slightly more active against the three substrates, and the material extracted at pH 9·1 slightly less active, than the material extracted at pH. 7·0.

Examination of Trichomonas foetus *extracts for enzyme activity: enzymes which act on glycoside substrates*

Galactosidases. The dialysed cell-free *T. foetus* filtrate was examined for its action on the α- and β-galactosides listed in Table 2. The mixtures after incubation were examined chromatographically for the liberation of galactose. A chromatogram of the *T. foetus* preparation without added substrate showed only a very faint spot with the benzidine–trichloroacetic acid spray reagent; the R_F of this spot was identical with that of glucose. Hydrolysis of all the galactoside substrates tested was observed, with the exception of methyl β-D-galactofuranoside, which was not attacked.

In addition to the galactosides listed in Table 2 the *T. foetus* extract hydrolysed the chromogenic

Table 2. *Hydrolysis of α- and β-D-galactosides by cell-free extracts of* T. foetus

Solutions (1%, w/v) of the substrates were incubated overnight at 37° with an equal volume of a 1% (w/v) solution of *T. foetus* extract dissolved in phosphate buffer (pH 7). ++, Complete hydrolysis; +, partial hydrolysis; −, no hydrolysis.

Substrate	Unheated *T. foetus* extract	*T. foetus* extract heated at 55° for 30 min.
Methyl α-D-galactopyranoside	+	−
Phenyl α-D-galactopyranoside	+	−
Methyl β-D-galactopyranoside	+	+
Methyl β-D-galactofuranoside	−	−
Melibiose	++	−
Lactose	++	+
O-α-D-Galactosyl-(1→6)-*N*-acetylglucosamine	++	−
O-α-D-Galactosyl-(1→6)-*N*-acetylgalactosamine	++	−
O-α-D-Galactosyl-(1→6)-D-galactose	++	−
O-β-D-Galactosyl-(1→3)-*N*-acetylglucosamine	++	.
O-β-D-Galactosyl-(1→4)-*N*-acetylglucosamine	++	++
O-β-D-Galactosyl-(1→6)-*N*-acetylglucosamine	++	.
O-β-D-Galactosyl-(1→6)-galactose	++	+

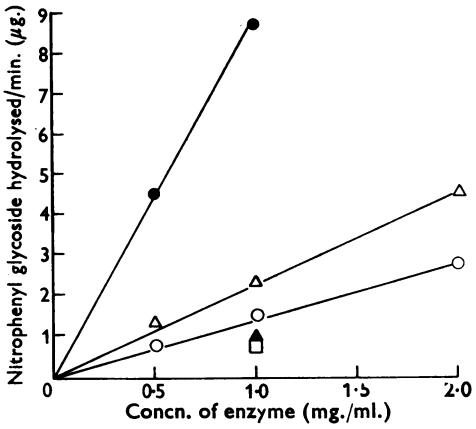

Fig. 1. Hydrolysis of nitrophenyl glycosides by cell-free extracts of *T. foetus*. Substrates: ○, *o*-nitrophenyl α-D-galactoside; △, *o*-nitrophenyl β-D-galactoside; ●, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide; ▲, *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide; □, *p*-nitrophenyl α-L-rhamnoside. Reaction mixtures: substrate (0·5 ml.), buffer solution (pH 7·0) (2·5 ml.) and enzyme solution (0·5 ml.) mixed together in optical cell. Optical density measured at 420 mμ at 1 min. intervals for a total of 5 min.

substrates *o*-nitrophenyl α- and β-D-galactosides. The relationship between the concentration of the *T. foetus* preparation and the activity of the α- and β-galactosidases, with the nitrophenyl galactosides as substrates, is shown in Fig. 1. Considerable variations in the activity of the galactosidases was observed in enzyme preparations obtained from different batches of organisms; the α-galactosidase activity, however, never exceeded, and was frequently considerably weaker than, the β-galactosidase activity. Owing to the ease with which the hydrolysis of the nitrophenyl glycosides can be measured the experiments involving galactosidases described below were carried out mainly with these substrates.

N-Acetylhexosaminidases. Solutions (1%, w/v) of methyl *N*-acetyl-α- and -β-D-glucosaminide, methyl *N*-acetyl-α-D-galactosaminide and *NN'*-diacetylchitobiose were incubated for 18 hr. at 37° with an equal volume of a dialysed solution of *T. foetus* extract (1%, w/v). As measured by the liberation of *N*-acetylglucosamine, 5% hydrolysis of methyl *N*-acetyl-α-glucosaminide and 42% hydrolysis of methyl *N*-acetyl-β-glucosaminide occurred under these conditions. Methyl *N*-acetyl-α-galactosaminide was hydrolysed to the extent of 30% as measured by the release of *N*-acetylgalactosamine. The β-1:4-linked *N*-acetylglucosamine disaccharide, *NN'*-diacetylchitobiose, was examined chromatographically for the liberation of *N*-acetylglucosamine and was found to be extensively hydrolysed. The enzyme preparation therefore contained a weak *N*-acetyl-α-glucosaminidase, an *N*-acetyl-β-glucosaminidase and an *N*-acetyl-α-galactosaminidase.

The liberation of *p*-nitrophenol from the chromogenic substrates *p*-nitrophenyl *N*-acetyl-β-glucosaminide and *p*-nitrophenyl *N*-acetyl-β-galactosaminide (Fig. 1) confirmed the presence of an *N*-acetyl-β-glucosaminidase in the *T. foetus* extract and showed that it contained, in addition, an *N*-acetyl-β-galactosaminidase.

Fucosidases. Fucose-containing compounds of known structure and enzyme in phosphate buffer (pH 7) were incubated together for 18 hr. at 37° and the resulting mixtures were examined chromatographically for the presence of free fucose. The results are shown in Table 3. In confirmation of earlier results (Watkins, 1955) no hydrolysis of either methyl α- or β-fucopyranosides or fucofuranosides was observed. Similarly, no free

Table 3. *Hydrolysis of fucose-containing compounds by cell-free extracts of* T. foetus

Equal volumes of 1% (w/v) solutions of fucose-containing compounds and *T. foetus* extract in phosphate buffer (pH 7) incubated together for 18 hr. at 37°. + +, Complete hydrolysis; +, partial hydrolysis; −, no hydrolysis.

Substrate	Nature of α-fucosyl linkage	Unheated *T. foetus* extract	*T. foetus* extract heated at 55° for 30 min.
Methyl α-L-fucopyranoside	.	−	.
Methyl β-L-fucopyranoside	.	−	.
Methyl α-L-fucofuranoside	.	−	.
Methyl β-L-fucofuranoside	.	−	.
O-α-L-Fucosyl-(1→2)-D-galactose	.	+ +	+ +
Fucosidolactose	(-(1→2)-D-galactose)	+ +	.
Lacto-*N*-fucopentaose I	(-(1→2)-D-galactose)	+ +	.
Lacto-*N*-fucopentaose II	(-(1→4)-*N*-acetylglucosamine)	+ +	+ +
Lactodifucotetraose	{-(1→2)-D-galactose} {-(1→3)-D-glucose }	+ +	+
O-α-L-Fucosyl-(1→2)-L-fucose	.	−	.
O-α-L-Fucosyl-(1→3)-L-fucose	.	−	.
O-α-L-Fucosyl-(1→4)-L-fucose	.	−	.

fucose was detected in the products from the action of *T. foetus* on the three α-linked fucose disaccharides from fucoidin. The chromatograms from the (1→2) and (1→3) compounds, however, each showed, in addition to the unchanged material, a spot, absent from the controls, with an R_F similar to that of fucosyl-(1→4)-fucose which may have arisen by enzymic transfer of the fucosyl linkage. The oligosaccharides from milk, however, were completely broken down into their component sugars, thus demonstrating that the three different types of α-fucosyl linkage in these compounds are all susceptible to attack by the enzymes in the *T. foetus* preparation.

Other glycosidases. In addition to the examination of the *T. foetus* extract for glycosidases which might play some part in the enzymic disruption of the blood-group substrates, the action of the preparation on the following substrates was tested: methyl-α- and -β-D-arabinosides, maltose, cellobiose, *p*-nitrophenyl α-L-rhamnoside and *p*-nitrophenyl α-D-mannoside. The treated methyl glycosides and disaccharides were examined chromatographically for the liberation of free sugars. Maltose and cellobiose were both partly hydrolysed but no hydrolysis of the arabinosides was observed. Liberation of *p*-nitrophenol from the rhamnoside substrate occurred on addition of the enzyme preparation (Fig. 1) but there was no hydrolysis of *p*-nitrophenyl α-D-mannoside.

Stability of Trichomonas foetus *enzymes*

Storage. Cell-free extracts from *T. foetus* which have been dried from the frozen state retain their activity against the blood-group substrates, *o*-nitrophenyl α- and β-galactosides and *p*-nitrophenyl β-*N*-acetylglucosaminide, for several months when the preparations are stored *in vacuo* over phosphorus pentoxide at −10°. Gradual loss of activity occurs, however, under these conditions, especially of the enzymes which destroy the activity of the A and Le^a blood-group substances.

Dialysis. Dialysis of the extracts for 1 week at 0–4° against frequent changes of normal saline or phosphate buffer (pH 6·8) does not lead to any greater loss in activity against the blood-group substrates than occurs in undialysed preparations which have stood for the same length of time at 0–4°. Dialysis against distilled water results in considerable loss of enzymic activity when the subsequent tests are carried out in a salt-free system. The activity can, however, be restored to its original level by the addition of normal saline or phosphate buffer (pH 6·8) to the test system. As a standard procedure the cell-free extracts were dialysed for 48 hr. against frequent changes of distilled water and dried from the frozen state. The preparations were stored *in vacuo* at −10° and reconstituted when required in phosphate buffer (pH 6·8).

Heat stability. Samples of the *T. foetus* extract were heated for 30 min. at various temperatures and tested for enzyme activity. The minimum amounts of A-, B-, H- and Le^a-substances giving complete inhibition of agglutination after treatment with the heated enzyme preparations are given in Table 4. The enzymes acting on the A-, B- and H-substrates retained a considerable percentage of their original activity after treatment at 55°, but were completely inactivated at 70°. Considerable inactivation of the enzyme acting on Le^a specific structures occurred at 55°. The enzyme acting on the property of the B-substance measured by immune rabbit anti-B serum was more readily destroyed by heat than was the specificity which is detected by human anti-B serum.

The activities of the α- and β-galactosidases, *N*-acetyl-β-glucosaminidase and *N*-acetyl-α-galactosaminidase in the heated preparations are shown in Table 5. The activity of the α-galactosidase, as measured by its action on *o*-nitrophenyl α-D-galactoside, is destroyed very readily by heat whereas the α-galactosaminidase, as measured by its action on methyl *N*-acetyl-α-galactosaminide, retains 88% of its activity after 30 min. heating at 55°.

The *T. foetus* preparation heated to 55° for 30 min. was tested for its action on some of the galactosides listed in Table 2. Chromatographic examination of the reaction products showed that activity against all the α-galactoside substrates was lost after this treatment. β-Galactosidase activity was retained, but in most instances hydrolysis was less extensive than with the unheated extract.

The action of the enzyme preparation on the liberation of fucose from α-L-fucosyl-(1→2)-D-galactose and lacto-*N*-fucopentaose II was unchanged on heating the extract to 55° (Table 3). Only partial hydrolysis of lactodifucotetraose occurred with the heated *T. foetus* preparation, and the chromatogram showed, in addition to fucose and traces of galactose and glucose, a spot with an R_F similar to that of the trisaccharide fucosidolactose.

Chromatographic examination of the mixtures obtained after the *T. foetus* preparation heated to 55° had acted on methyl α- and β-*N*-acetylglucosaminides and *NN'*-diacetylchitobiose showed that these three compounds were hydrolysed by the preparation; the intensity of the spots with Ehrlich's reagent did not appear to differ greatly from that given by the reaction products obtained with the unheated *T. foetus* extract.

pH *stability*. Enzyme solutions (1% w/v) after thorough dialysis against distilled water were mixed with equal volumes of buffer solutions at pH 3, 5, 7, 9 and 11. The mixtures were left for 1 hr. at room temperature (21°), each solution was then readjusted to pH 7 and the activity of the enzymes acting on A-, B- and H-substances was determined by the standard method. The H-decomposing enzyme retained full activity over the range pH 5–9, showed some loss of activity at pH 3 and was completely inactivated at pH 11. The

Table 4. *Heat stability of enzymes which destroy the serological activity of the A-, B-, H- and Le^a-substances*

Blood-group substance	Test serum	Minimum amount of blood-group substance (μg./ml.) giving complete inhibition after treatment with enzyme heated for 30 min. at 20°	45°	55°	60°	65°	70°	100°
A	Human anti-A	63	63	63	4	2	1	1
B	Human anti-B	125	125	125	16	2	0·5	0·5
	Immune rabbit anti-B	63	32	16	2	1	1	1
H	Immune rabbit anti-H	>250	>250	125	8	2	2	2
Le^a	Human anti-Le^a	>250	>250	4	1	1	1	1

Table 5. *Heat stability of α- and β-galactosidases,* N-*acetyl-β-glucosaminidase and* N-*acetyl-α-galactosaminidase*

Substrate	Percentage enzyme activity remaining after treatment of *T. foetus* extract for 30 min. at 37°	45°	55°	65°	100°
o-Nitrophenyl α-D-galactoside*	5	0	0	0	0
o-Nitrophenyl β-D-galactoside*	40	11	8	4	0
p-Nitrophenyl *N*-acetyl-β-glucosaminide*	85	74	42	3	0
Methyl *N*-acetyl-α-galactosaminide†	—	—	88	40	0

* Activity determined by measuring rate of liberation of nitrophenol.
† Activity determined by measuring amount of *N*-acetylgalactosamine liberated after incubation of equal volumes of 1% (w/v) solutions of enzyme and substrate for 18 hr. at 37°.

Table 6. pH *stability of α- and β-galactosidases and* N-*acetyl-β-glucosaminidase in cell-free* T. foetus *extracts*

Substrate	Percentage enzyme activity remaining after treatment of *T. foetus* extract for 1 hr. at pH 3	5	7	9	11
o-Nitrophenyl α-D-galactoside	0	55	100	100	10
o-Nitrophenyl β-D-galactoside	0	72	100	51	0
p-Nitrophenyl *N*-acetyl-β-glucosaminide	3	76	100	99	0

enzymes acting on A- and B-substances were less stable and showed complete loss of activity at pH 3 and 11 and some loss of activity at pH 9. The pH stabilities of the α- and β-galactosidases and *N*-acetyl-β-glucosaminidase in the same enzyme preparations are given in Table 6.

pH *optima*

The influence of pH on the activity of the enzymes acting on the blood-group substrates was determined by incubation of the enzyme–substrate mixtures for 2 hr. at 37° in the presence of buffer solutions at various pH values between 4 and 9. At the end of the incubation time each mixture was adjusted to pH 7, and the solutions were heated at 100° for 10 min. and titrated for blood-group activity. The limitations of the agglutination-inhibition test, which allows only changes of the order of 50 % in the concentration of the substrate to be detected, do not enable a precise determination of the pH optima to be made. The results (Table 7) indicated, however, that maximum inactivation of A-, B-, H- and Le^a-substances was obtained in the pH range 6–7.

The pH optima for the α- and β-galactosidases and *N*-acetyl-β-glucosaminidase were determined with the nitrophenyl glycosides as substrates. The rates of liberation of nitrophenol from these substrates at different pH values could not be determined directly owing to the variations in the proportions of the coloured tautomeric forms of *o*- and *p*-nitrophenol which occur with changes in pH (Lederberg, 1950). The following procedure was therefore used: Buffer solution (5 ml.) was mixed with the substrate (0·5 ml.) and enzyme solution (0·1 ml.) added. The mixtures were allowed to stand for 10 min. at room temperature and 1 ml. of 1 M-sodium carbonate solution was then added to stop the reaction and to convert all the liberated nitrophenol into the coloured form. The intensity of colour was measured at 420 mμ. The β-galactosidase had a pH optimum in the range 6·3–6·6 (Fig. 2), the α-galactosidase showed maximum activity in the range pH 5·7–6·6 (Fig. 2) and the

Table 7. pH *optima of the enzymes in* T. foetus *extracts acting on A-, B-, H- and Le^a-substances*

For details see text.

pH of enzyme–substrate mixture during incubation	Minimum amount of blood-group substance (μg./ml.) giving complete inhibition			
	A	B	H	Le^a
4·0	2	2	32	4
5·4	8	8	63	8
6·0	16	32	63	8
7·0	16	32	125	16
7·4	16	16	63	8
8·0	8	2	32	1
9·1	2	1	4	1
Control (boiled enzyme)	1	0·5	2	1

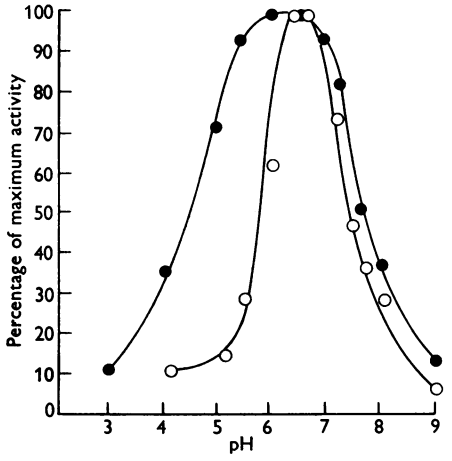

Fig. 2. Effect of pH on the hydrolysis of *o*-nitrophenyl α-D-galactoside (●), and *o*-nitrophenyl β-D-galactoside (○). Reaction mixtures: buffer solution (5 ml.), substrate (0·5 ml.) and 1 % enzyme solution (0·1 ml.). After 10 min. at room temp. the reaction was stopped by the addition of 1 ml. of M-sodium carbonate solution.

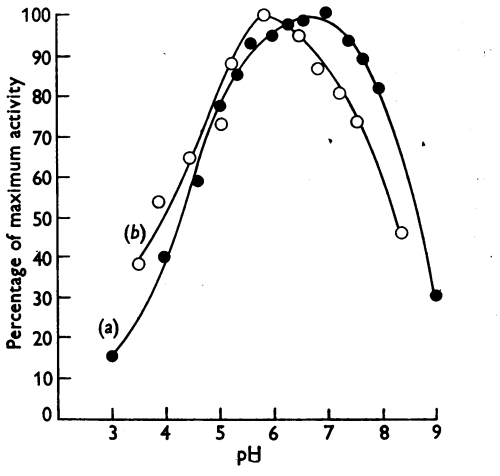

Fig. 3. (*a*) Effect of pH on the hydrolysis of *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (●). Reaction mixture: buffer solution (5 ml.), substrate (0·5 ml.) and 1 % enzyme solution (0·1 ml.). After 10 min. at room temp. reaction was stopped by the addition of 1 ml. of M-sodium carbonate solution. (*b*) Effect of pH on the hydrolysis of methyl *N*-acetyl-α-D-galactosaminide (○). Equal volumes of substrate (0·5 %), buffer and enzyme solution (1 %, w/v) were incubated at 37° for 8 hr. At the end of the incubation period the solution was adjusted to pH 7 and the free *N*-acetylgalactosamine estimated.

N-acetyl-β-glucosaminidase had optimum pH 7·0–7·2 (Fig. 3). The optimum pH for the liberation of *N*-acetylgalactosamine from methyl *N*-acetyl-α-galactosaminide was 6·0 (Fig. 3).

Inhibition of enzyme activity

Sugars. The specific inhibition by simple sugars of the enzymes in cell-free extracts of *T. foetus* acting on A-, B- and H-substances was previously reported (Watkins & Morgan, 1955*a*). The destruction of 1 mg. (1 ml.) of A-, B- and H-substances by the enzymes in 10 mg. (1 ml.) of the *T. foetus* extract was specifically, and, as far as can be determined by the serological inhibition test, completely inhibited by 20 mg. (1 ml.) of *N*-acetylgalactosamine, D-galactose and L-fucose respectively. *N*-Acetylglucosamine and a considerable number of other sugars failed to inhibit the enzymes. The action of simple sugars on the activity of the enzymes hydrolysing *o*-nitrophenyl α- and β-galactosides, *p*-nitrophenyl *N*-acetyl-β-glucosaminide and *p*-nitrophenyl *N*-acetyl-β-galactosaminide was therefore examined. The concentration of the sugars relative to the amount of *T. foetus* extract was the same as that used in the experiments on the blood-group substrates. The results are summarized in Table 8.

The aldonolactones have been shown to inhibit a wide variety of glycosidases (Levvy, 1952; Conchie, 1954; Conchie & Levvy, 1957). The action of D-galactono-γ-lactone on the enzymes which decompose the A-, B- and H-substances and on the α- and β-galactosidases and *N*-acetyl-β-glucosaminidase was therefore tested. The inactivation of 1 mg. (1 ml.) of the blood-group substrates by 10 mg. (1 ml.) of the *T. foetus* preparation was not inhibited by the addition of up to 200 mg. (1 ml.) of D-galactono-γ-lactone dissolved in buffer at pH 7. At a similar concentration ratio of enzyme, substrate and inhibitor only 5% inhibition of the hydrolysis of *p*-nitrophenyl *N*-acetyl-β-glucosaminide was observed. The α- and β-galactosidases in the *T. foetus* preparation acting on the *o*-nitrophenyl α- and β-galactosides were both susceptible to inhibition by D-galactono-γ-lactone; the percentage inhibition of the two enzyme activities with different concentrations of D-galactono-γ-lactone is shown in Fig. 4.

Inorganic ions. The inhibiting effects of the following inorganic compounds on the enzymes acting on A-, B- and H-substances, *o*-nitrophenyl α- and β-galactosides, *p*-nitrophenyl *N*-acetyl-β-glucosaminide and methyl *N*-acetyl-α-galactosaminide were examined: lead nitrate, cobalt chloride, zinc chloride, copper sulphate, manganese chloride, silver nitrate and mercuric chloride. Each solution (0·05%) was added to an equal volume of a 1% (w/v) solution of *T. foetus* extract. All reactions were carried out in phosphate buffer at pH 7 and some precipitation of the heavy-metal ions occurred. The A-, B- and H-decomposing

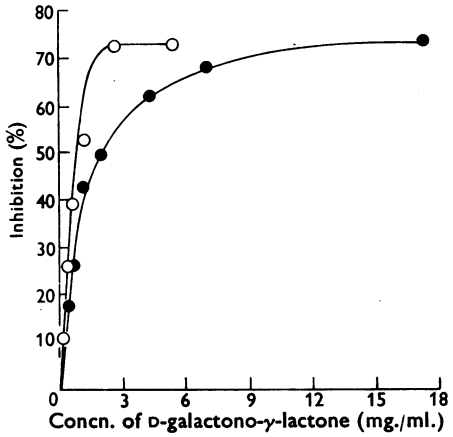

Fig. 4. Inhibition of hydrolysis of *o*-nitrophenyl α-D-galactoside (○), and of *o*-nitrophenyl β-D-galactoside (●), by D-galactono-γ-lactone. Reaction mixtures: galactonolactone solution (0·5 ml.), 1% (w/v) enzyme solution (0·1 ml.), buffer solution (pH 7) (2·4 ml.), substrate (0·5 ml.).

Table 8. *Inhibition of α- and β-galactosidases,* N-*acetyl-β-glucosaminidase and* N-*acetyl-β-galactosaminidase by simple sugars*

For details see text.

Sugar added	Inhibition of enzyme activity (%)			
	α-Galactosidase*	β-Galactosidase†	*N*-Acetyl-β-glucosaminidase‡	*N*-Acetyl-β-galactosaminidase§
L-Fucose	0	0	0	—
D-Galactose	22	2	0	—
N-Acetylglucosamine	20	0	36	37
N-Acetylgalactosamine	0	0	70	68
D-Glucose	22	0	0	—

* Substrate: *o*-nitrophenyl α-D-galactoside.
† Substrate: *o*-nitrophenyl β-D-galactoside.
‡ Substrate: *p*-nitrophenyl *N*-acetyl-β-glucosaminide.
§ Substrate: *p*-nitrophenyl *N*-acetyl-β-galactosaminide.

Table 9. *Inhibition of α- and β-galactosidases, N-acetyl-β-glucosaminidase and N-acetyl-α-galactosaminidase with metal ions*

For details see text.

Inhibitor	Inhibition of enzyme activity (%)			
	α-Galactosidase*	β-Galactosidase†	*N*-Acetyl-β-glucosaminidase‡	*N*-Acetyl-α-galactosaminidase§
Copper sulphate	21	29	23	26
Manganese chloride	0	6	0	11
Mercuric chloride	100	66	100	57
Silver nitrate	100	100	100	56
Lead nitrate	5	3	0	—
Cobalt chloride	0	4	2	—
Zinc chloride	0	0	0	—

* Substrate: *o*-nitrophenyl α-D-galactoside.
† Substrate: *o*-nitrophenyl β-D-galactoside.
‡ Substrate: *p*-nitrophenyl *N*-acetyl-β-glucosaminide.
§ Substrate: methyl *N*-acetyl-α-galactosaminide.

enzymes were completely inhibited by mercuric chloride and partially inhibited by copper sulphate. Complete inhibition of the A- and B-enzymes was also observed with silver nitrate but this compound did not detectably reduce the activity of the H-enzyme. Lead nitrate, cobalt chloride, zinc chloride and manganese chloride failed to inhibit the enzymes acting on the blood-group substrates under the conditions used. The action of the inorganic ions on the liberation of nitrophenol from the nitrophenyl glycosides and on the liberation of *N*-acetylgalactosamine from methyl *N*-acetyl-α-galactosaminide is shown in Table 9.

DISCUSSION

The nature of the chemical changes in the water-soluble blood-group substances brought about by the crude *T. foetus* preparations has not been extensively investigated as it was considered that information relating to the specificity differences between the substances could be obtained only when purified enzyme preparations were available. An examination of the material which becomes diffusible after enzyme action, however, showed that loss of serological specificity was accompanied by the liberation from each substance of fucose, galactose and *N*-acetylhexosamine. It seems probable therefore that a proportion of all the component sugars, namely L-fucose, D-galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine, is split off from each substance and that the *T. foetus* preparation must contain several enzymes capable of splitting glycosidic bonds of the type present in the blood-group substances. Ryley (1955), in studies on the carbohydrate metabolism of *T. foetus*, demonstrated the presence in cell-free preparations of maltase, amylase and phosphorylase. The present investigation has shown that the *T. foetus* extracts contain enzymes capable of hydrolysing a range of low-molecular-weight glycosides of known structure. As it appeared possible that some of the glycosidases might be identified with enzymes primarily responsible for the loss of serological specificity of the blood-group substances, or with those that cause further decomposition of the mucopolysaccharide molecules after loss of specificity has occurred, some of their properties have been compared with those of the enzymes which decompose the blood-group substances.

Watkins & Morgan (1955*a*) suggested that the enzyme which destroys the serological properties of B-substance has a galactosidase specificity. This suggestion was based on the capacity of galactose to inhibit the enzymic decomposition of B-substance, coupled with the absence of any appreciable inhibition by a number of other sugars. Preliminary experiments with a purified B-enzyme from *T. foetus* supported this idea, since destruction of the B-substance was shown to be accompanied by the liberation mainly of galactose with only traces of fucose and *N*-acetylhexosamine (Watkins, 1956). Moreover, the quantitative precipitation-inhibition experiments of Kabat & Leskowitz (1955) indicated that a terminal α-galactosyl residue is involved in B-specificity. If, therefore, the function of the enzymes which destroy blood-group activity is envisaged as the removal of the terminal sugar of the structural units that confer on the blood-group substances their specific serological characters, then the B-enzyme would presumably have an α-galactosidase specificity. The *T. foetus* preparation was found to hydrolyse a wide range of low-molecular-weight α- and β-galactopyranoside structures (Table 2 and Fig. 1). Although the differences in the method of testing for loss of blood-group activity and for the action on the low-molecular-weight glycosides do not enable strictly quantitative comparisons to be made, certain differences

have emerged between the properties of the enzymes acting on the low-molecular-weight α-galactosides and on B-substance which suggest that they are not identical. The α-galactosidase activity against all the substrates tested, for example, was destroyed when the preparation was heated for 30 min. at 55°, whereas the B-enzyme retained its activity unimpaired after such treatment. Inhibition of α-galactosidase activity was observed with D-galactose when *o*-nitrophenyl galactoside was used as substrate, but glucose and *N*-acetylglucosamine gave a similar degree of inhibition, whereas these sugars are without action on the B-enzyme. Similarly D-galactono-γ-lactone, which gave powerful inhibition of both α- and β-galactosidases, did not inhibit the destruction of B-serological activity. These results do not exclude the possibility that the B-enzyme has an α-galactosidase specificity, but suggest that if this attribution is correct the enzyme must have strict specificity requirements with regard to the aglycon which are not fulfilled by any of the low-molecular-weight α-galactoside substrates used in the present experiments.

Agglutination- and precipitation-inhibition tests with simple sugars have suggested that an *N*-acetyl-α-galactosaminoyl structure plays an important part in A-specificity (Morgan & Watkins, 1953; Kabat & Leskowitz, 1955; Côté & Morgan, 1956). The inhibition by *N*-acetylgalactosamine of the enzymic decomposition of A-substance (Watkins & Morgan, 1955*a*), taken in conjunction with the serological findings, suggested that the enzyme responsible for loss of A-activity is an α-galactosaminidase. The results so far obtained do not enable the conclusion to be reached that the enzyme acting on methyl *N*-acetyl-α-galactosaminide is identical with the enzyme acting on A-substance, but the pH optima, temperature-stability and susceptibility to inhibition by metal ions of the two enzyme activities are similar. When further purification procedures have been carried out it will be possible to determine whether these two properties remain closely associated and whether differences will be revealed by inhibition and activation experiments carried out with a wider variety of reagents.

Earlier attempts to demonstrate the presence of fucosidases in the *T. foetus* preparations by means of their action on methyl α- and β-fucopyranosides and furanosides were unsuccessful (Watkins, 1955). The rapid and extensive liberation of fucose from all the blood-group substances by the action of the crude *T. foetus* preparation indicated, however, that an enzyme with fucosidase specificity must be present. Examination of the action of the enzyme preparation on fucose-containing disaccharides and oligosaccharides of known structure has now shown that the *T. foetus* extracts contain an enzyme, or enzymes, capable of hydrolysing α-L-fucosyl linkages. Thus fucose was readily liberated when it was joined by an α-1:2-linkage to galactose as in *O*-α-L-fucosyl-(1→2)-D-galactose, fucosidolactose, lacto-*N*-fucopentaose I or lactodifucotetraose, by an α-1:4-linkage to *N*-acetylglucosamine, as in lacto-*N*-fucopentaose II or by an α-1:3-linkage to glucose as is the second fucose linkage in lactodifucotetraose (Table 3). The preparation failed to hydrolyse the three fucose-containing disaccharides from fucoidin, however, despite the fact that they contain the same three linkages as are found in the oligosaccharides from milk. This finding supports the conclusion that the nature of the aglycon is very important and that neither a methyl group nor a second fucose unit meets the specificity requirements of the enzymes.

The demonstration of enzymes capable of splitting fucosyl bonds is of especial interest from the point of view of the H- and Le^a-enzymes. Serological inhibition tests with simple sugars have suggested that, although the two serological characters are quite distinct, α-L-fucosyl units form an important part of the specific structures which determine both H (Watkins & Morgan, 1952; Morgan & Watkins, 1953) and Le^a (Watkins & Morgan, 1957) blood-group specificity. The inhibition by L-fucose of the enzymes in *T. foetus* preparations which destroy the serological activity of H-substance (Watkins & Morgan, 1955*a*) and Le^a-substance (Watkins & Morgan, 1957) has supported these findings and has led to the assumption that the enzymes have α-fucosidase specificity. The greater lability of the Le^a-enzyme than of the H-enzyme to both heat and storage suggests that different enzymes are involved in the serological inactivation of the two substances. Further experiments will need to be carried out before it can be determined whether the enzymes that have been shown to split α-L-fucosyl bonds are identical with the enzymes acting on either the H- or Le^a-specific structures.

The powerful *N*-acetyl-β-glucosaminidase, and the weaker *N*-acetyl-β-galactosaminidase found in the *T. foetus* extracts would not be expected to play any part in the destruction of the serological properties of A-, B-, H- and Le^a-substances if the specificities predicted for the enzymes bringing about these changes are correct. However, structures containing *N*-acetyl-β-glucosaminoyl linkages exist in A-substance (Côté & Morgan, 1956) and probably also occur in the other group substances. It is possible therefore that the β-glucosaminidase could effect changes in the molecules other than those leading to loss of serological specificity and the removal of this enzyme during purification of the crude *T. foetus* extract would

seem desirable. The finding that both the *N*-acetyl-β-glucosaminidase and the *N*-acetyl-β-galactosaminidase are inhibited by *N*-acetylgalactosamine to a greater extent than by *N*-acetylglucosamine shows that caution must be used in assuming that the sugars which are the most powerful inhibitors are of necessity those which are the products of enzyme action. Since the activity of the enzyme preparation towards *o*-nitrophenyl *N*-acetyl-β-glucosaminide is about ten times the activity towards *o*-nitrophenyl *N*-acetyl-β-galactosaminide it does not seem likely that the enzyme is acting on both substrates by virtue of a galactosaminidase specificity. It should perhaps be emphasized, however, that the specificities suggested for the A-, B-, H- and Le^a-enzymes were based on the enzyme-inhibition results, taken in conjunction with a considerable amount of evidence from serological inhibition studies which had pointed in each instance to the importance of a certain sugar for serological specificity.

The exact changes involved in the development of a degree of H-specificity by A- and B-substances after enzyme treatment have not been determined; the observation promises to be of importance, however, both from the point of view of structural studies and also in consideration of the biosynthesis of the blood-group materials. The finding that A-, B- and H-substances develop the capacity to cross-react with anti-type XIV pneumococcus horse serum after treatment with the *T. foetus* enzymes is also of considerable interest. The action of enzyme preparations from *Cl. welchii* (Buchanan *et al.* 1957) or *Cl. tertium* (Kabat, 1958) does not result in the development of type XIV cross-reactivity; indeed, any pre-existing capacity of the blood-group materials to precipitate with the antiserum is destroyed by these enzymes. The presence of β-linked galactosyl-*N*-acetylglucosamine units in both the type XIV polysaccharide and the blood-group mucopolysaccharides is believed to provide the basis for the cross-reactivity of the blood-group substances with anti-type XIV serum (see Kabat, 1956, 1958; Watkins & Morgan, 1956). The three possible types of linkage for such a disaccharide unit are β-1:3, β-1:4 and β-1:6. The *T. foetus* preparation contains enzymes capable of hydrolysing each of these linkages and it is therefore surprising that these structures in the blood-group substances, or in the type XIV polysaccharide (Watkins, 1953), are apparently not attacked.

In the present study no account has been taken of the proteolytic enzymes in extracts of *T. foetus*. There is much evidence to suggest that the specificity of the water-soluble blood-group substances is determined to a large extent by the configuration of certain of the carbohydrate constituents; a role for the amino acid components cannot be excluded, however, and recent unpublished observations of Mr A. Pusztai and Professor W. T. J. Morgan in this Department indicate that certain proteolytic enzymes can cause an extensive but limited loss of serological activity. When purified *T. foetus* enzyme preparations are available an examination will be made for peptidases and their possible action on the blood-group mucopolysaccharides.

Future progress in the use of the enzymes which decompose blood-group substances will depend on the discovery of efficient methods for the separation and purification of the complex mixtures of enzymes present in crude extracts of *T. foetus*.

SUMMARY

1. Cell-free extracts of *Trichomonas foetus* contain enzymes which destroy the serological properties of human water-soluble A-, B-, H- and Le^a-blood-group substances. The optimum for each enzyme is between pH 6 and 7.

2. Dialysis of the *T. foetus* extracts against distilled water results in loss of enzyme activity on the blood-group substrates, but inactivation is reversed by the addition of salts. The enzymes acting on A-, B- and H-substances are stable to heating for 30 min. at 55° but considerable inactivation of the Le^a enzyme occurs under these conditions.

3. Incubation of A-, B-, H- and Le^a-substances for 18 hr. at 37° with the *T. foetus* extract results in the production of about 20% of reducing sugars and a liberation of fucose, galactose and *N*-acetylhexosamine.

4. In addition to its action on the blood-group substrates the *T. foetus* preparation hydrolyses compounds of known structure containing α- or β-galactoside, α- or β-glucoside, *N*-acetyl-α- or -β-glucosaminide, *N*-acetyl-α- or -β-galactosaminide, α-L-fucoside or α-L-rhamnoside linkages.

5. The possible relationships between the enzymes acting on the low-molecular-weight substrates and those acting on the blood-group substrates are discussed.

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